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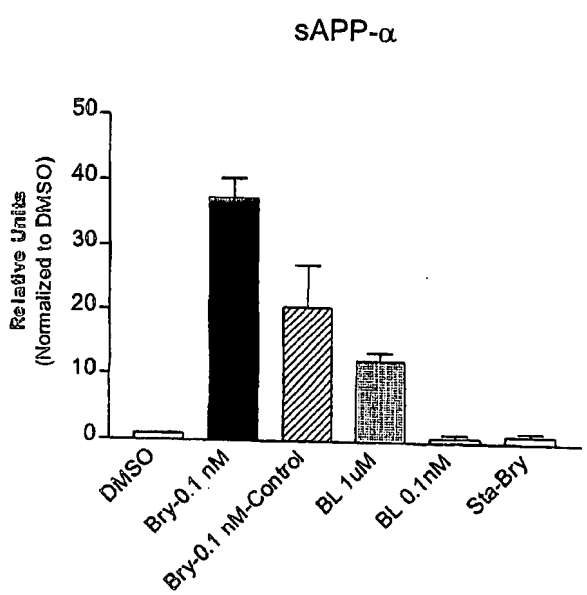
(54) Title: PKC ACTIVATION AS A MEANS FOR ENHANCING sAPPα SECRETION AND IMPROVING COGNITION USING BRYOSTATIN TYPE COMPOUNDS



(57) Abstract: The present invention relates to compositions and methods to modulate α-secretase and/or to improve cognitive ability. The invention further relates the improved/enhanced cognitive ability in diseased individuals, particularly Alzheimer's Disease patients, and treatment thereof through increased sAPP production. Macrocyclic lactones (i.e. bryostatin class and neristatin class) are compounds preferred for use with the present composition. The present invention also provides methods for increasing the generation of non-amyloidogenic soluble APP comprising the activation of protein kinase C (PKC) by administering an effective amount of PKC activator(s).

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PKC Activation as a Means for Enhancing sAPPα Secretion and Improving Cognition Using Bryostatin Type Compounds

BACKGROUND OF THE INVENTION

(i) Field of the Invention

The present invention relates to the modulation of α-secretase and cognitive enhancement. The invention further relates to compounds for treatment of conditions associated with amyloid processing such as Alzheimer's Disease and compositions for the treatment of such conditions.

(ii) Background of the Invention

Various disorders and diseases exist which affect cognition. Cognition can be generally described as including at least three different components: attention, learning, and memory. Each of these components and their respective levels affect the overall level of a subject's cognitive ability. For instance, while Alzheimer's Disease patients suffer from a loss of overall cognition and thus deterioration of each of these characteristics, it is the loss of memory that is most often associated with the disease. In other diseases patients suffer from cognitive impairment that is more predominately associated with different characteristics of cognition. For instance Attention Deficit Hyperactivity Disorder (ADHD), focuses on the individual's ability to maintain an attentive state. Other conditions include general dementias associated with other neurological diseases, aging, and treatment of conditions that can cause deleterious effects on mental capacity, such as cancer treatments, stroke/ischemia, and mental retardation.

Cognition disorders create a variety of problems for today's society. Therefore, scientists have made efforts to develop cognitive enhancers or cognition activators. The cognition enhancers or activators that have been developed are generally classified to include nootropics, vasodilators, metabolic enhancers, psychostimulants, cholinergic agents, biogenic amine drugs, and neuropeptides. Vasodilators and metabolic enhancers (e.g. dihydroergotoxine) are mainly effective in the cognition disorders induced by cerebral vessel ligation-ischemia; however, they are ineffective in clinical use and with other types of cognition disorders. Of

treat the symptoms of Alzheimer's Disease patients which, while theoretically plausible and effective in mice tests, have been shown to cause severe adverse reactions in humans.

As a result, use of the cholinergic pathway for the treatment of cognitive impairment, particularly in Alzheimer's Disease, has proven to be inadequate. Additionally, the current treatments for cognitive improvement are limited to specific neurodegenerative diseases and have not proven effective in the treatment of other cognitive conditions.

Alzheimer's disease is associated with extensive loss of specific neuronal subpopulations in the brain with memory loss being the most universal symptom. (Katzman, R. (1986) New England Journal of Medicine 314:964). Alzheimer's disease is well characterized with regard to neuropathological changes. However, abnormalities have been reported in peripheral tissue supporting the possibility that Alzheimer's disease is a systemic disorder with pathology of the central nervous system being the most prominent. (Connolly, G., Fibroblast models of neurological disorders: fluorescence measurement studies, Review, TiPS Vol. 19, 171-77 (1998)). For a discussion of Alzheimer's disease links to a genetic origin and chromosomes 1, 14, and 21 see St. George-Hyslop, P. H., et al., Science 235:885 (1987);Tanzi, Rudolph et al., The Gene Defects Responsible for Familial Alzheimer's Disease, Review, Neurobiology of Disease 3, 159-168 (1996); Hardy, J., Molecular genetics of Alzheimer's disease, Acta Neurol Scand: Supplement 165: 13-17 (1996).

While cellular changes leading to neuronal loss and the underlying etiology of the disease remain under investigation the importance of APP metabolism is well established. The two proteins most consistently identified in the brains of patients with Alzheimer's disease to play a role in the physiology or pathophysiology of brain are β-amyloid and tau. (See Selkoe, D., Alzheimer's Disease: Genes, Proteins, and Therapy, Physiological Reviews, Vol. 81, No. 2, 2001). A discussion of the defects in β-amyloid protein metabolism and abnormal calcium homeostasis and/or calcium activated kinases. (Etcheberrigaray et al., Calcium responses are altered in fibroblasts from Alzheimer's patients and pre-symptomatic PS1 carriers: a potential tool for early diagnosis, Alzheimer's Reports, Vol. 3, Nos. 5 & 6, pp. 305-312

other isoforms are activated by phospholipid and diacylglycerol but are not dependent on Ca^{2+}. All isoforms encompass 5 variable (V1-V5) regions, and the α, β, γ isoforms contain four (C1-C4) structural domains which are highly conserved. All isoforms except PKCα, β and γ lack the C2 domain, and the λ, η and isoforms also lack nine of two cysteine-rich zinc finger domains in C1 to which diacylglycerol binds. The C1 domain also contains the pseudosubstrate sequence which is highly conserved among all isoforms, and which serves an autoregulatory function by blocking the substrate-binding site to produce an inactive conformation of the enzyme (House et al., *Science*, 238, 1726 (1987)).

Because of these structural features, diverse PKC isoforms are thought to have highly specialized roles in signal transduction in response to physiological stimuli (Nishizuka, *Cancer*, 10, 1892 (1989)), as well as in neoplastic transformation and differentiation (Glazer, *Protein Kinase C*, J. F. Kuo, ed., Oxford U. Press (1994) at pages 171-198). For a discussion of known PKC modulators see PCT/US97/08141, U.S. Patent Nos. 5,652,232; 6,043,270; 6,080,784; 5,891,906; 5,962,498; 5,955,501; 5,891,870 and 5,962,504.

In view of the central role that PKC plays in signal transduction, PKC has proven to be an exciting target for the modulation of APP processing. It is well established that PKC plays a role in APP processing. Phorbol esters for instance have been shown to significantly increase the relative amount of non-amyloidogenic soluble APP (sAPP) secreted through PKC activation. Activation of PKC by phorbol ester does not appear to result in a direct phosphorylation of the APP molecule, however. Irrespective of the precise site of action, phorbol-induced PKC activation results in an enhanced or favored α-secretase, non-amyloidogenic pathway. Therefore PKC activation is an attractive approach for influencing the production of non-deleterious sAPP and even producing beneficial sAPP and at the same time reduce the relative amount of Aβ peptides. Phorbol esters, however, are not suitable compounds for eventual drug development because of their tumor promotion activity. (Ibarreta, et al., Benzolactam (BL) enhances sAPP secretion in fibroblasts and in PC12 cells, NeuroReport, Vol. 10, No. 5&6, pp 1035-40 (1999)).

There is increasing evidence that the individual PKC isozymes play different,

compounds and compositions of the present invention are selected from macrocyclic lactones of the bryostatin and neristatin class.

In another aspect the invention relates to macrocyclic lactone compounds, compositions and methods that modulate α-secretase activity. Of particular interest are the bryostatin and neristatin class compounds, and of further interest is bryostatin-1.

Another aspect of the invention relates to the bryostatin and neristatin class compounds, as a PKC activator, to alter conditions associated with amyloid processing in order to enhance the α-secretase pathway to generate soluble α-amyloid precursor protein (αAPP) so as to prevent β-amyloid aggregation and improve/enhance cognitive ability. Such activation, for example, can be employed in the treatment of Alzheimer's Disease, particularly, bryostatin-1.

In another aspect, the invention relates to a method for treating plaque formation, such as that associated with Alzheimer's Disease, and improving/enhancing the cognitive state of the subject comprising administering to the subject an effective amount of a bryostatin or neristatin class compound. In a more preferred embodiment the compound is bryostatin-1.

Another aspect of the invention relates to a composition for treating plaque formation and improving/enhancing cognitive ability comprising: (i) a macrocyclic lactone in an amount effective to elevate soluble β-amyloid, generate soluble αAPP and prevent β-amyloid aggregation; and (ii) a pharmaceutically effective carrier. In a preferred embodiment the composition is used to improve/enhance cognitive ability associated with Alzheimer's Disease. The macrocyclic lactone is preferably selected from the bryostatin or neristatin class compounds, particularly bryostatin-1.

In one embodiment of the invention the activation of PKC isoenzymes results in improved cognitive abilities. In one embodiment the improved cognitive ability is memory. In another embodiment the improved cognitive ability is learning. In another embodiment the improved cognitive ability is attention. In another embodiment PKC's isoenzymes are activated by a macrocyclic lactone (i.e. bryostatin class and neristatin class). In particular, bryostatin-1 through 18 and

In another embodiment bryostatin-1 is used in combination with a non-bryostatin class compound to improve cognitive ability and reduce side effects.

In another embodiment of the invention, the modulation of PKC through macrocyclic lactones (i.e. bryostatin class and neristatin class) is used *in vitro* for the testing of conditions associated with Alzheimer's Disease. The *in vitro* use may include for example, the testing of fibroblast cells, blood cells, or the monitoring of ion channel conductance in cellular models.

In a preferred embodiment of the invention the compounds and compositions are administered through oral and/or injectable forms including intravenously and intraventricularly.

The present invention therefore provides a method of treating impaired memory or a learning disorder in a subject, the method comprising administering thereto a therapeutically effective amount of one of the present compounds. The present compounds can thus be used in the therapeutic treatment of clinical conditions in which memory defects or impaired learning occur. In this way memory and learning can be improved. The condition of the subject can thereby be improved.

The compositions and methods have utility in treating clinical conditions and disorders in which impaired memory or a learning disorder occurs, either as a central feature or as an associated symptom. Examples of such conditions which the present compounds can be used to treat include Alzheimer's disease, multi-infarct dementia and the Lewy-body variant of Alzheimer's disease with or without association with Parkinson's disease; Creutzfeld-Jakob disease and Korsakow's disorder.

The compositions and methods can also be used to treat impaired memory or learning which is age-associated, is consequent upon electro-convulsive therapy or which is the result of brain damage caused, for example, by stroke, an anesthetic accident, head trauma, hypoglycemia, carbon monoxide poisoning, lithium intoxication or a vitamin deficiency.

The compounds have the added advantage of being non-tumor promoting and already being involved in phase II clinical trials.

graph units are relative to the vehicle, DMSO, alone. Bryostatin was significantly ($p < 0.01$, Tukey's post test) more potent than another PKC activator, BL, at the same (0.1 nM) concentration. Pre-treatment (rightmost bar) with staurosporin (100 nM) completely abolished the effect of bryostatin (0.1 nM). Bryostatin was also effective in enhancing secretion in two control cell lines, although to a lesser extend than in the AD cell line (hatched bar);

Fig. 1(b) illustrates the effect of different concentrations of Bryostatin-1 on sAPPα secretion over a time course - Secretion is clearly near enhanced by 15 min incubation (bryostatin 0.1 nM) and near maximal at 160 incubation, remaining elevated up to 3 h. Bryostatin at lower, 0.01 nM, was much slower but had about the same effect on secretion after 120 min incubation;

Fig. 1(c) illustrates the secretion of sAPPα under various experimental conditions and cells lines through a Western blot representation of sAPP-α in human fibroblasts;

Fig. 2 illustrates the effect of different concentrations of Bryostatin-1 on the PKCα isozyme.

Fig. 3 illustrates the amount of time required for treated rats verse controls to learn a water maze - The learning curves in the Morris Water Maze show that bryostatin (i.v.c.) improved the performance of the animals as evidenced by reduction of the escape latency from early trials;

Fig. 4(a) illustrates the amount of time control rats spent swimming in the different quadrants - Both controls and treated animals show retention of preference for the target quadrant (see also figure 4(b);

Fig. 4(b) illustrates the amount of time treated rats spent swimming in the different quadrants.

Fig. 4(c) illustrates the different between the amount of time the treated rats spent in target quadrant compared to control rats - Treated animals showed improved retention compared to controls;

Fig. 5 (a) illustrates PKC translocation in human fibroblasts with bar graphs showing the ratios between the immunoreactivity (normalized by total protein content) of the membrane bound PKC (P=particulate) and the immunoreactivity

Fig. 14 illustrates the decreased percent of plaques found in treated animal compared to controls following Thioflavin S staining.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Memory loss and impaired learning ability are features of a range of clinical conditions. For instance, loss of memory is the most common symptom of dementia states including Alzheimer's disease. Memory defects also occur with other kinds of dementia such as multi-infarct dementia (MID), a senile dementia caused by cerebrovascular deficiency, and the Lewy-body variant of Alzheimer's disease with or without association with Parkinson's disease, or Creutzfeld-Jakob disease. Loss of memory is a common feature of brain-damaged patients. Brain damage may occur, for example, after a classical stroke or as a result of an anesthetic accident, head trauma, hypoglycemia, carbon monoxide poisoning, lithium intoxication, vitamin (B1, thiamine and B12) deficiency, or excessive alcohol use or Korsakow's disorder. Memory impairment may furthermore be age-associated; the ability to recall information such as names, places and words seems to decrease with increasing age. Transient memory loss may also occur in patients, suffering from a major depressive disorder, after electro-convulsive therapy (ECT). Alzheimer's disease is in fact the most important clinical entity responsible for progressive dementia in ageing populations, whereas hypoxia/stroke is responsible for significant memory defects not related to neurological disorders.

Individuals with Alzheimer's disease are characterized by progressive memory impairments, loss of language and visuospatial skills and behavior deficits (McKhann et al., 1986, Neurology, 34:939-944). The cognitive impairment of individuals with Alzheimer's disease is the result of degeneration of neuronal cells located in the cerebral cortex, hippocampus, basal forebrain and other brain regions. Histologic analyses of Alzheimer's disease brains obtained at autopsy demonstrated the presence of neurofibrillary tangles (NFT) in perikarya and axons of degenerating neurons, extracellular neuritic (senile) plaques, and amyloid plaques inside and around some blood vessels of affected brain regions. Neurofibrillary tangles are

production of fragments that later aggregate forming the amyloid deposits characteristic of Alzheimer's disease (AD), known as senile or AD plaques. Thus, APP processing is an early and key pathophysiological event in AD.

Three alternative APP processing pathways have been identified. The previously termed "normal" processing involves the participation of an enzyme that cleaves APP within the Aβ sequence at residue Lys16 (or between Lys16 and Leu17; APP770 nomenclature), resulting in non-amyloidogenic fragments: a large N-terminus ectodomain and a small 9 kDa membrane bound fragment. This enzyme, yet to be fully identified, is known as α-secretase. Two additional secretases participate in APP processing. One alternative pathway involves the cleavage of APP outside the Aβ domain, between Met671 and Asp672 (by β-secretase) and the participation of the endosomal-lysomal system. An additional cleavage site occurs at the carboxyl-terminal end of the Aβ portion, within the plasma membrane after amino acid 39 of the Aβ peptide. The secretase (γ) action produces an extracellular amino acid terminal that contains the entire Aβ sequence and a cell-associated fragment of ~6kDa. Thus, processing by β and γ secretases generate potential amyloidogenic fragments since they contain the complete Aβ sequence. Several lines of evidence have shown that all alternative pathways occur in a given system and that soluble Aβ may be a "normal product." However, there is also evidence that the amount of circulating Aβ in CSF and plasma is elevated in patients carrying the "Swedish" mutation. Moreover, cultured cells transfected with this mutation or the APP_{717} mutation, secrete larger amounts of Aβ. More recently, carriers of other APP mutations and PS1 and PS2 mutations have been shown to secrete elevated amounts of a particular form, long (42-43 amino acids) Aβ.

Therefore, although all alternative pathways may take place normally, an imbalance favoring amyloidogenic processing occurs in familial and perhaps sporadic AD. These enhanced amyloidogenic pathways ultimately lead to fibril and plaque formation in the brains of AD patients. Thus, intervention to favor the non-amyloidogenic, α-secretase pathway effectively shifts the balance of APP processing towards a presumably non-pathogenic process that increases the relative amount of sAPP compared with the potentially toxic Aβ peptides.

brain tissues of AD patients. The levels and/or activity of this enzyme(s) were introduced in brains and fibroblasts from AD patients (Cole et al., 1988; Van Huynh et al., 1989; Govoni et al., 1993; Wang et al., 1994). Studies using immunoblotting analyses have revealed that of the various PKC isozymes, primarily the α isoform was significantly reduced in fibroblasts (Govoni et al., 1996), while both α and β isoforms are reduced in brains of AD patients (Shimohama et al., 1993; Masliah et al., 1990). These brain PKC alterations might be an early event in the disease process (Masliah et al., 1991). It is also interesting to note that PKC activation appears to favor nonamyloidogenic processing of the amyloid precursor protein, APP (Buxbaum et al., 1990; Gillespie et al., 1992; Selkoe, 1994; Gandy & Greengard, 1994; Bergamashi et al., 1995; Desdouits et al., 1996; Efhimiopoulus et al., 1996). Thus, both PKC and K^+ channel alterations coexist in AD, with peripheral and brain expression in AD.

The link between PKC and K^+ channel alterations has been investigated because PKC is known to regulate ion channels, including K^+ channels and that a defective PKC leads to defective K^+ channels. This is important not only for the modulation of APP, but also for the role PKC and K^+ channels play in memory establishment and recall. (e.g., see Alkon et al., 1988; Covarrubias et al., 1994; Hu et al., 1996) AD fibroblasts have been used to demonstrate both K^+ channels and PKC defects (Etcheberrigaray et al., 1993; Govoni et al., 1993, 1996). Studies also show, fibroblasts with known dysfunctional K^+ channels treated with PKC activators restore channel activity as monitored by the presence/absence of TEA-induced calcium elevations. Further, assays based on tetraethylammonium chloride (TEA)-induced $[Ca^{2+}]$ elevation have been used to show functional 113pS K^+ channels that are susceptible to TEA blockade (Etcheberrigaray et al., 1993, 1994; Hirashima et al., 1996). Thus, TEA-induced $[Ca^{2+}]$ elevations and K^+ channel activity observed in fibroblasts from control individuals are virtually absent in fibroblasts from AD patients (Etcheberrigaray et al., 1993; Hirashima et al., 1996). These studies demonstrate that the use of PKC activators can restore the responsiveness of AD fibroblast cell lines to the TEA challenge. Further, immunoblot evidence from these studies demonstrate that this restoration is related to a preferential participation of

for example, Hollister, L. E., 1990, Pharmacopsychiat., 23, (Suppl II) 33-36). The available animal models of memory loss and impaired learning involve measuring the ability of animals to remember a discrete event. These tests include the Morris Water Maze and the passive avoidance procedure. In the Morris Water Maze, animals are allowed to swim in a tank divided into four quadrants, only one of which has a safety platform beneath the water. The platform is removed and the animals are tested for how long they search the correct quadrant verse the incorrect quadrants. In the passive avoidance procedure the animal remembers the distinctive environment in which a mild electric shock is delivered and avoids it on a second occasion. A variant of the passive avoidance procedure makes use of a rodent's preference for dark enclosed environments over light open ones. Further discussion can be found in Crawley, J. N., 1981, Pharmacol. Biochem. Behav., 15, 695-699; Costall, B. et al, 1987, Neuropharmacol., 26, 195-200; Costall, B. et al, 1989, Pharmacol. Biochem. Behav., 32, 777-785; Barnes, J. M. et al, 1989, Br. J. Pharmacol., 98 (Suppl) 693P; Barnes, J. M. et al, 1990, Pharmacol. Biochem. Behav., 35, 955-962.

The use of the word, "normal" is meant to include individuals who have not been diagnosis with or currently display diminished or otherwise impaired cognitive function. The different cognitive abilities may be tested and evaluated through known means well established in the art, including but not limited to tests from basic motor-spatial skills to more complex memory recall testing. Non-limiting examples of tests used for cognitive ability for non-primates include the Morris Water Maze, Radial Maze, T Maze, Eye Blink Conditioning, Delayed Recall, and Cued Recall while for primate subjects test may include Eye Blink, Delayed Recall, Cued Recall, Face Recognition, Minimental, and ADAS-Cog. Many of these tests are typically used in the mental state assessment for patients suffering from AD. Similarly, the evaluation for animal models for similar purposes with well describe in the literature.

Of particular interest are macrocyclic lactones (i.e. bryostatin class and neristatin class) that act to stimulate PKC. Of the bryostatin class compounds, bryostatin-1 has been shown to activate PKC and proven to be devoid of tumor

1343-46 (1987); Phase II Trial of Bryostatin 1 in Patients with Relapse Low-Grade Non-Hodgkin's Lymphoma and Chronic Lymphocytic Leukemia, Varterasian et al., Clinical Cancer Research, Vol. 6, pp. 825-28 (2000); and Review Article: Chemistry and Clinical Biology of the Bryostatins, Mutter et al., Bioorganic & Medicinal Chemistry 8, pp. 1841-1860 (2000).

Macrocyclic lactones, including the bryostatin class, represent known compounds, originally derived from *Bugula neritina L.* While multiple uses for macrocyclic lactones, particularly the bryostatin class are known, the relationship between macrocyclic lactones and cognition enhancement was previously unknown.

The examples of the compounds that may be used in the present invention include macrocyclic lactones (i.e. bryostatin class and neristatin class compounds). While specific embodiments of these compounds are described in the examples and detailed description, it should be understood that the compounds disclosed in the references and derivatives thereof could also be used for the present compositions and methods.

As will also be appreciated by one of ordinary skill in the art, macrocyclic lactone compounds and their derivatives, particularly the bryostatin class, are amenable to combinatorial synthetic techniques and thus libraries of the compounds can be generated to optimize pharmacological parameters, including, but not limited to efficacy and safety of the compositions. Additionally, these libraries can be assayed to determine those members that preferably modulate α-secretase and/or PKC.

Combinatorial libraries high throughput screening of natural products and fermentation broths has resulted in the discovery of several new drugs. At present, generation and screening of chemical diversity is being utilized extensively as a major technique for the discovery of lead compounds, and this is certainly a major fundamental advance in the area of drug discovery. Additionally, even after a "lead" compound has been identified, combinatorial techniques provide for a valuable tool for the optimization of desired biological activity. As will be appreciated, the subject reactions readily lend themselves to the creation of combinatorial libraries of compounds for the screening of pharmaceutical, or other biological or medically-

Other compounds which may be included by admixture are, for example, medically inert ingredients, e.g. solid and liquid diluent, such as lactose, dextrose, saccharose, cellulose, starch or calcium phosphate for tablets or capsules, olive oil or ethyl oleate for soft capsules and water or vegetable oil for suspensions or emulsions; lubricating agents such as silica, talc, stearic acid, magnesium or calcium stearate and/or polyethylene glycols; gelling agents such as colloidal clays; thickening agents such as gum tragacanth or sodium alginate, binding agents such as starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinylpyrrolidone; disintegrating agents such as starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuff; sweeteners; wetting agents such as lecithin, polysorbates or laurylsulphates; and other therapeutically acceptable accessory ingredients, such as humectants, preservatives, buffers and antioxidants, which are known additives for such formulations.

Liquid dispersions for oral administration may be syrups, emulsions or suspensions. The syrups may contain as carrier, for example, saccharose or saccharose with glycerol and/or mannitol and/or sorbitol. In particular a syrup for diabetic patients can contain as carriers only products, for example sorbitol, which do not metabolize to glucose or which metabolize only a very small amount to glucose. The suspensions and the emulsions may contain a carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose or polyvinyl alcohol.

Suspensions or solutions for intramuscular injection may contain, together with the active compound, a pharmaceutically acceptable carrier such as sterile water, olive oil, ethyl oleate, glycols such as propylene glycol and, if desired, a suitable amount of lidocaine hydrochloride. Solutions for intravenous injection or infusion may contain a carrier, for example, sterile water that is generally Water for Injection. Preferably, however, they may take the form of a sterile, aqueous, isotonic saline solution. Alternatively, the present compounds may be encapsulated within liposomes. The present compounds may also utilize other known active agent delivery systems.

provide evidence that another PKC activator, benzolactam, causes a significant increase in sAPPα and reduction of Aβ40 *in vivo*.

All books, articles, or patents references herein are incorporated by reference to the extent not inconsistent with the present disclosure. The present invention will now be described by way of examples, which are meant to illustrate, but not limit, the scope of the invention.

Example I:

Cell Culture

Cultured skin fibroblasts were obtained from the Coriell Cell Repositories and grown using the general guidelines established for their culture with slight modifications (Cristofalo & Carptentier, 1988; Hirashima et al., 1996). The culture medium in which cells were grown was Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (Biofluids, Inc.). Fibroblasts from control cell lines (AC), cases AG07141 and AG06241, and a familial AD (FAD) case (AG06848) were utilized.

PKC Activators

The different tissue distributions, the apparently distinctive roles of different isozymes, and the differential involvement in pathology make it important to use pharmacological tools that are capable of preferentially targeting specific isozymes (Kozikowski et al., 1997; Hofmann, 1997). Recent research in the medicinal chemistry field has resulted in the development of several PKC activators, for instance different benzolactams and pyrollidinones. However, the currently studied bryostatin PKC activator not only has the benefit of providing isospecific activity, but also does not suffer from the set back of the previously used PKC activator, such as being tumor promoting. The bryostatin competes for the regulatory domain of PKC and engages in very specific hydrogen bond interactions within this site. Additional information on the organic chemistry and molecular modeling of this compound can be found throughout the literature.

Treatment

substrate (Vector Laboratories) per the manufacturer's instructions. The band intensities were quantified by densitometry using a BioRad GS-800 calibrated scanning densitometer and Multianalyst software (BioRad).

sAPP - Determinations/Measurements of sAPPα

The concentration of secreted APP was measured using conventional immunoblotting techniques, with minor modifications the protocol. Precipitated protein extracts from each dish/treatment were loaded to freshly prepared 10% acrylamide Tris·HCl minigels and separated by SDPAGE. The volume of sample loaded was corrected for total cell protein per dish. Proteins were then electrophoretically transferred to PVDF membranes. Membranes were saturated with 5% non-fat dry milk to block non-specific binding. Blocked membranes were incubated overnight at 4 °C with the commercially available antibody 6E10 (1:500), which recognizes sAPP-alpha in the conditioned medium (SENETEK). After washing, the membranes were incubated at room temperature with horseradish peroxidase conjugated anti-mouse IgG secondary antibody (Jackson's Laboratories). The signal was then detected using enhanced chemiluminescence followed by exposure of Hyperfilm ECL (Amersham). The band intensities were quantified by densitometry using a BioRad GS-800 calibrated scanning densitometer and Multianalyst software (BioRad).

As shown in Fig. 8 and 9, Bryostatin-1 elicits a powerful response, demonstrating the activation of PKC, It should be noted the activation of PKC is easily detectable 30 minutes after delivery, following a dose of only 0.1 nM of bryostatin-1.

It is also interesting to consider the data in relation to APP metabolism and the effects of its sub-products. Studies have demonstrated that PKC activation increases the amount of ratio of non-amyloidogenic (soluble APP, presumably product of the secretase) vs. amyloidogenic (Aβ1-40 and/or Aβ1-42) secreted fragments (Buxbaum et al., 1990; Gillespie et al., 1992; Selkoe, 1994). Without wishing to be held to this theory, one could speculate that AD cells with low PKC would have an impaired secretion of sAPP and/or have increased proportion of

on identical schedule. On the fifth day, the platform was removed and the retention test was conducted. Animals' movements and escape latencies were recorded with an automatic tracking system. Learning was measured as the reduction of escape latency from trial to trial, which was significantly lower in the treated animals. Acquisition of memory was measured as time spent in the relevant quadrant (5^{th} day). Memory or retention was significantly enhanced in treated animals, compared to sham injection animals (see Figures 3 through 4(a)-4(c)). The rats treated with bryostatin-1 showed improved cognition over control rats within 2 days of treatment (see Figure 3). Bryostatin-1 is capable of being used at concentrations to improve cognition that are 300 to 300,000 times lower than the concentration used to treat tumors. The above example further shows that cognitive ability can be improved in non-diseased subjects as compared to other non-diseased subjects through the administration of bryostatin-1.

Because of the previously conducted safety, toxicology and phase II clinical studies for cancer, one can conclude that the use of PKC activators, particularly bryostatin-1, would be viewed as safe and that phase II studies for AD treatment/cognitive enhancement could be expedited. Furthermore, bryostatin-1's lipophilic nature provides increased blood brain barrier transport. The present invention would allow for intravenous, oral, intraventricullar, and other known methods of administration.

Test of sAPP secretion experiments, PKC activation experiments, and animal behavior experiments have shown that increases in sAPP secretion follow increased PKC activation and result in improved cognition in animal behavior studies.

Example III: Transgenic animals and *in vivo* studies

Transgenic mice carrying the V717I mutation were treated with BL (1mg/kg, i.p.; daily) from ~ 3 weeks of age (after weaning) for 17 weeks (n=4). The control group (n=4) received vehicle alone (Tween 20 1%, DMSO 25%, 74% PBS). Another experimental group consisted of 5-6 months old animals treated for 7 weeks. Subgroups of these animas were treated with BL 1mg/kg, daily (n=5); BL 10 mg/kg, daily (n=3; due to two deaths); BL 10 mg/kg, weekly (n=4; one death),

ELISA of amyloid peptides. Protein extracts were applied on reversed-phase columns (C18-Sep-pack cartridges; Waters Corporation, Milford, MA) and washed with increasing concentrations of acetonitrile (5, 25, and 50%) containing 0.1% trifluoroacetic acid. The last fraction contained the amyloid peptides and was dried *in vacuo* overnight and dissolved for measurements in ELISA. Sandwich ELISA for human Aβ40 and Aβ42 peptides was performed using the capture antiserum JRF/cAβ40/10 and 21F12, respectively, and they were developed with monoclonal antibodies JRFcAβtot/14hrpo and 3D6, respectively (Vanderstichele H, Van Kerschaver E, Hese C, Davidsson P, Buyse MA, Andreansen N, Minthon L, Wallin A, Blennow K, Vanmechelen E. Standardization of measurements of beta-amyloid (1-42) in cerebrospinal fluid and plasma. Amyloid 2000; 7: 245-258).

Standard general health assessment and open field were conducted in all animals prior to the biochemical assessments. In addition, a semi-quantitative *ad hoc* score was devised to measure abdominal contractions that followed the injections (+ = weak, ≤2 min; ++: strong, ≥min; +++: very strong, ≥1.2 min).

Example IV: Transgenic Animals and *in vivo* Studies Using Bryostatin

A second transgenic study using similar procedures/testing and protocol was performed using double transgenic mice carrying the V717I mutation and a Presenilin-1 (PS1) mutation, which causes accelerated amyloid formation, with the following major differences. Approximately 40 mice including both treated and controls were utilized. Treatment began at approximately 3 weeks of age and consisted of treatments with 40 μg/k.g. i.p. three times a week using Bryostatin-1. Controls were given vehicle alone. The treatment continued for approximately seven months before the morbidity rate of the non-treated animals necessitated termination of the experiment (See, Fig. 10). While behavioural differences between the treated and non-treated animals were not significant using water testing (See, Fig. 11), treated animals demonstrated decreases in soluble Aβ-40 (See, Fig. 12) and soluble Aβ-42 (See, Fig. 13). Additionally, the treated mice demonstrated an overall lower amount of total APP as show in Figure 14 where Thioflavin S staining shows a decrease in percent plaque load compared to controls.

Discussion of Above Experiments

different translocation than DMSO alone (not shown). The effect of 0.01 nM bryostatin was much less marked and slow, with a maximum P/S ratio value at 120 min incubation. Levels of translocation of other PKC isoenzymes were assessed at 30 min incubation with 0.1 nM bryostatin. Clear immunoreactivity was detected (both membrane-bound and cytosolic) with specific antibodies for ϵ, β and δ izoenzymes. The ratio S/P was higher in all cases than DMSO alone and comparable to the levels of PKC-α (Fig. 5b).

Behavior (MWM): The learning curve of the group receiving bryostatin was significantly faster than the control group. Escape latencies were clearly reduced from early trials and lower than the control group from trial 3. The quadrant preference test showed retention in both groups, but was significantly enhanced for the bryostatin treated group, compared to controls. Fig. 3-4(c) summarizes these results.

Transgenic animals: The transgenic animals treated with BL from 3 weeks of age for 17 weeks showed a significant increase in sAPP-α and a concomitant and proportional reduction in Aβ40 (Fig. 6 (a)-(b)). There were no differences in the amount of Aβ42, APP membrane-bound and total secreted sAPP (sAPPα + sAPPβ). Animals showed no differences in general health and weight gain was similar in both groups. Injections caused variable abdominal contractions (reversible) with similar frequency in both groups. The intensity was somewhat elevated in the BL-treated group (data not shown). In addition, BL treated animals showed an increase in open field test scores, without reaching statistical significance (not shown).

The animals treated later in life (6 months of age) and for a shorter period (~7 weeks) did not show any dramatic changes in terms of APP species. The general trend (small changes), however, was in the same direction as described for the longer-term treatment (previous section). There was slight increase in sAPPα in animals treated with BL 10 mg/kg (daily and weekly) and also in animals treated with LQ12 10 mg/kg, daily (Fig 7(b), solid bars). BL 1mg/kg (daily) and LQ12 10 mg/kg (weekly) had no effect (fig 5A, pattern bars). A slight decrease in Aβ40 was observed in animals treated with BL (n=5) and LQ12 (n=5), both 10 mg/kg, weekly

The results showing an improvement performance of normal rats in the MWM task after bryostatin administration (i.c.v.) demonstrate that PKC activation can cause cognitive enhancement as an added therapeutic effect. Additionally, secreted APP may by itself improve memory in normal and amnestic mice. These experiments and models demonstrate the PKC regulation, particularly through bryostatin-1 can result in an increase in sAPP and/or an improvement in memory. They also demonstrate that a regime which includes a PKC activator can be used to prevent build up of toxic fragments and prevent memory decline.

12. The method of claim 11, wherein the brain damage was caused by stroke, an anesthetic accident, head trauma, hypoglycemia, carbon monoxide poisoning, lithium intoxication or a vitamin deficiency.

13. The method of claim 1, wherein the PKC activator is administered in an amount effected to cause an increase in sAPP.

14. A method for altering cellular modulation of ion channels comprising administering a PKC activator in an amount effective for altering cellular modulation of ion channels and a pharmaceutically acceptable carrier.

15. The method of claim 14 wherein, said modulation is *in vivo* or *in vitro* modulation.

16. The method of claim 15, wherein said ion channel is a K^+ or Ca^{++} channel.

17. A method for treating neurotumors comprising administering macrocyclic lactone in an amount effective to treat said neurotumors and a pharmaceutically acceptable carrier.

18. The method of claim 17 wherein, the macrocyclic lactone is a bryostatin class or neristatin class compound.

19. A method for modulating sAPP comprising administering a macrocyclic lactone in an amount effective to modulate sAPP and a pharmaceutically acceptable carrier.

20. The method of claim 19 wherein, the macrocyclic lactone activates PKC.

21. The method of claim 19 wherein, the macrocyclic lactone is a bryostatin class or a neristatin class compound.

22. The method of claim 19 wherein, the macrocyclic lactone is bryostatin-1 through bryostatin 18, or neristatin-1.

23. A method for modulating α-secretase comprising administering a macrocyclic lactone in an amount effective to modulate α-secretase anda pharmaceutically suitable carrier.

34. A method for providing a neuroprotective effect for cell comprising administering a bryostatin or neristatin class compound in an amount effective to provided a neuroprotective effect for cells which suffer from a hypoxic event and a pharmaceutically acceptable carrier.

35. A method for providing a neuroprotective effect for cell comprising administering bryostatin-1 in an amount effective to provided a neuroprotective effect for cells which suffer from a hypoxic event and a pharmaceutically acceptable carrier.

36. A method for the reduction of amyloid plaque formation comprising administering bryostatin-1 in an amount effective to reduce amyloid plaque formation and a pharmaceutically acceptable carrier.

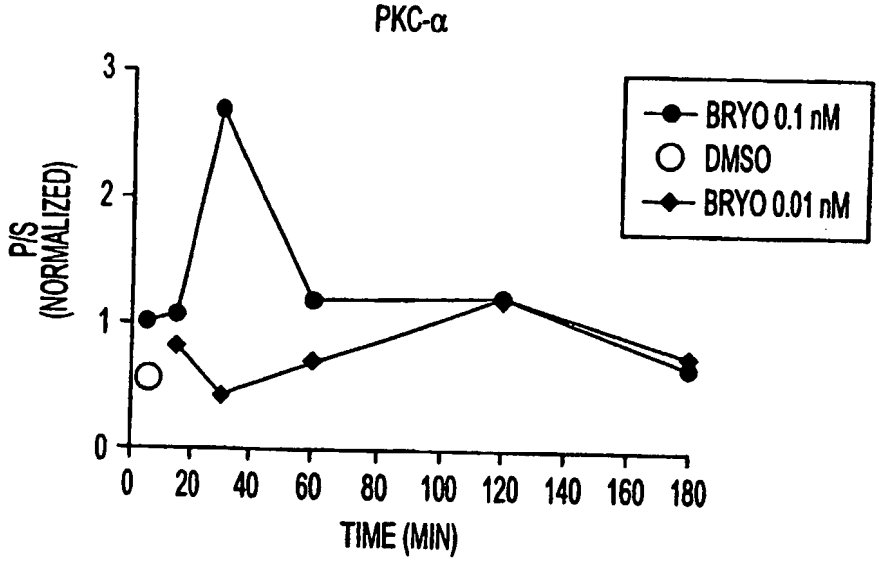

PKC-α
P/S (NORMALIZED)
3
2
1
0
0
20
40
60
80
100
120
140
160
180
TIME (MIN)
BRYO 0.1 nM
DMSO
BRYO 0.01 nM

FIG. 1B

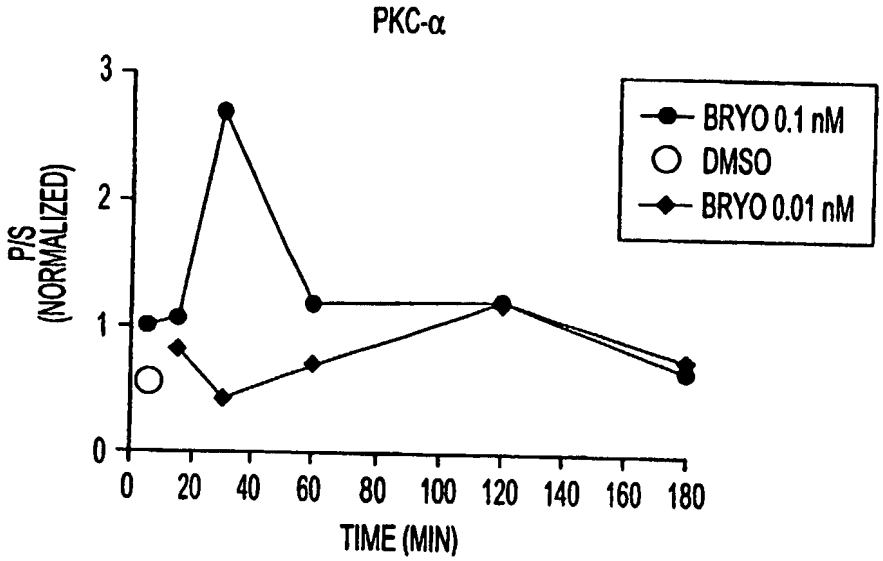
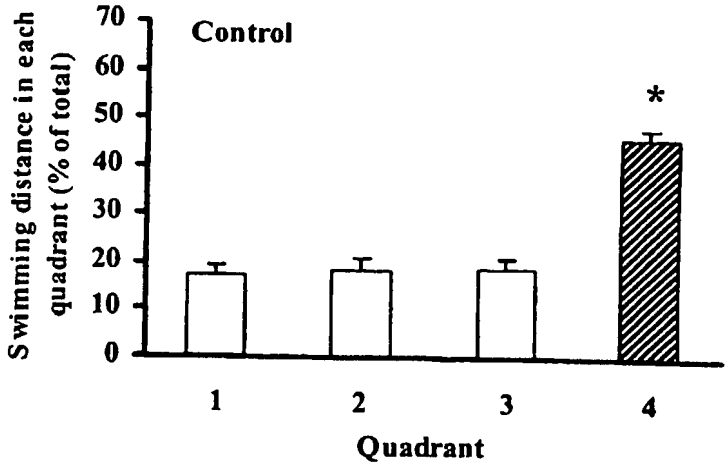
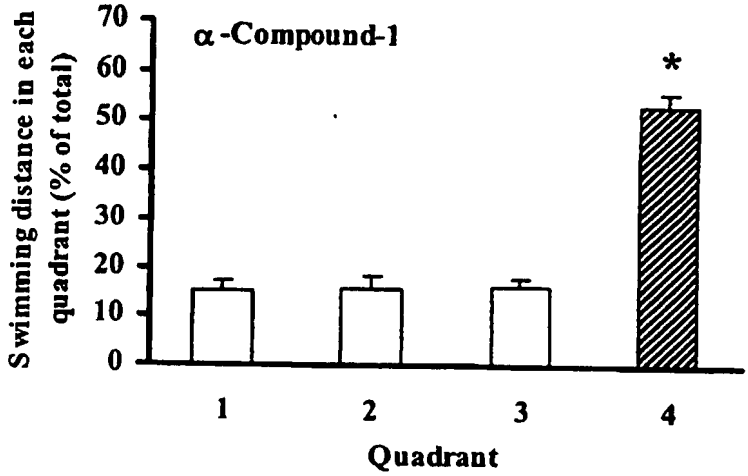

PKC-α
P/S (NORMALIZED)
3
2
1
0
0
20
40
60
80
100
120
140
160
180
TIME (MIN)
BRYO 0.1 nM
DMSO
BRYO 0.01 nM

FIG. 2

A


Control
Swimming distance in each quadrant (% of total)
70
60
50
40
30
20
10
0
*
1
2
3
4
Quadrant

B


α-Compound-1
Swimming distance in each quadrant (% of total)
70
60
50
40
30
20
10
0
*
1
2
3
4
Quadrant

C

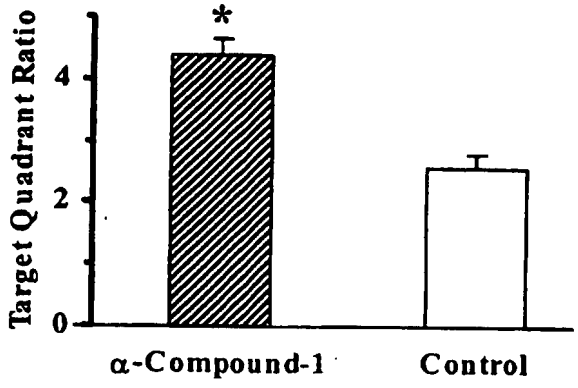

Target Quadrant Ratio
4
2
0
*
α-Compound-1
Control

FIG. 4

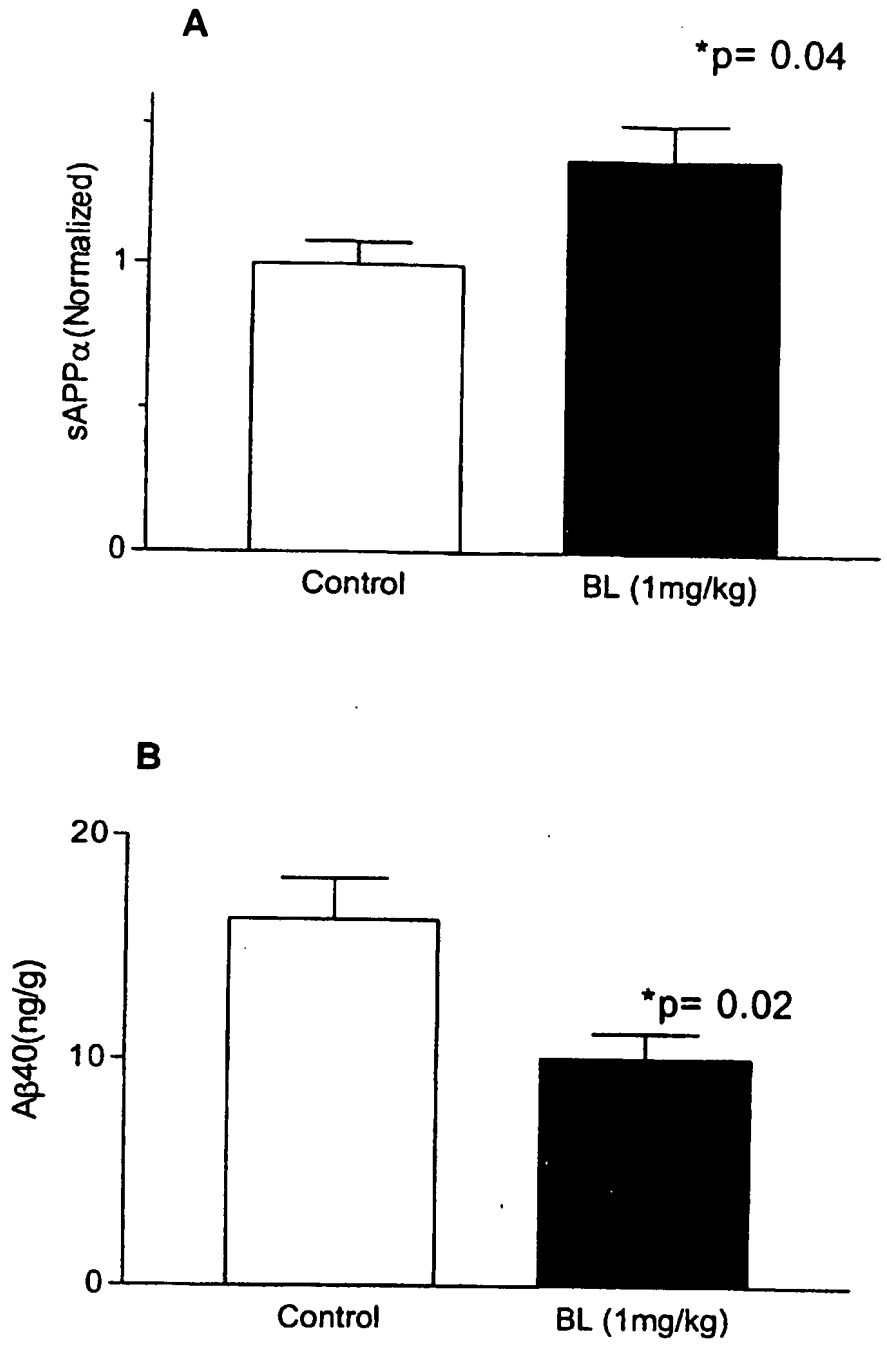

A
*p= 0.04
sAPPα(Normalized)
1
0
Control
BL (1mg/kg)
B
20
10
0
Aβ40(ng/g)
*p= 0.02
Control
BL (1mg/kg)

FIG. 6

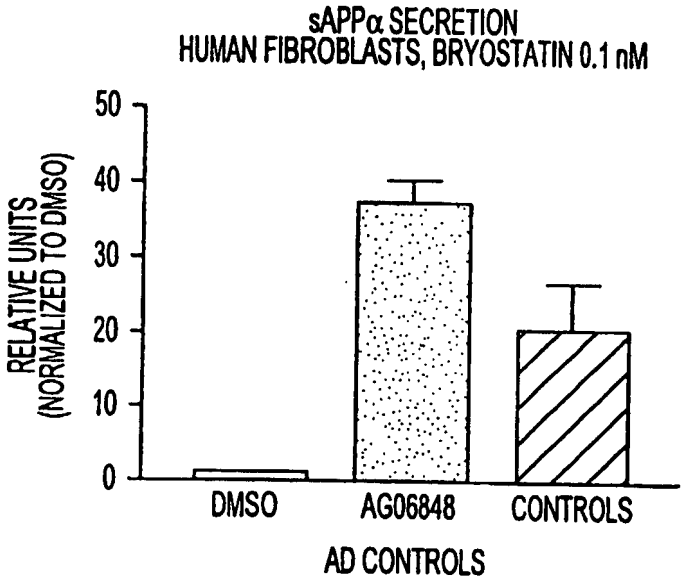

sAPPα SECRETION
HUMAN FIBROBLASTS, BRYOSTATIN 0.1 nM
RELATIVE UNITS
(NORMALIZED TO DMSO)
50
40
30
20
10
0
DMSO
AG06848
CONTROLS
AD CONTROLS

FIG. 8

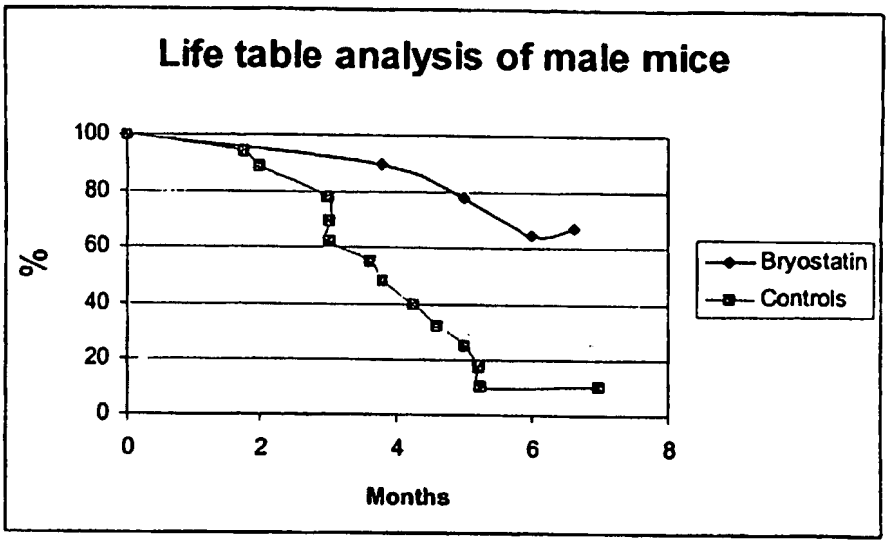

Life table analysis of male mice
100
80
60
40
20
0
%
0
2
4
6
8
Months
Bryostatin
Controls

FIG. 10

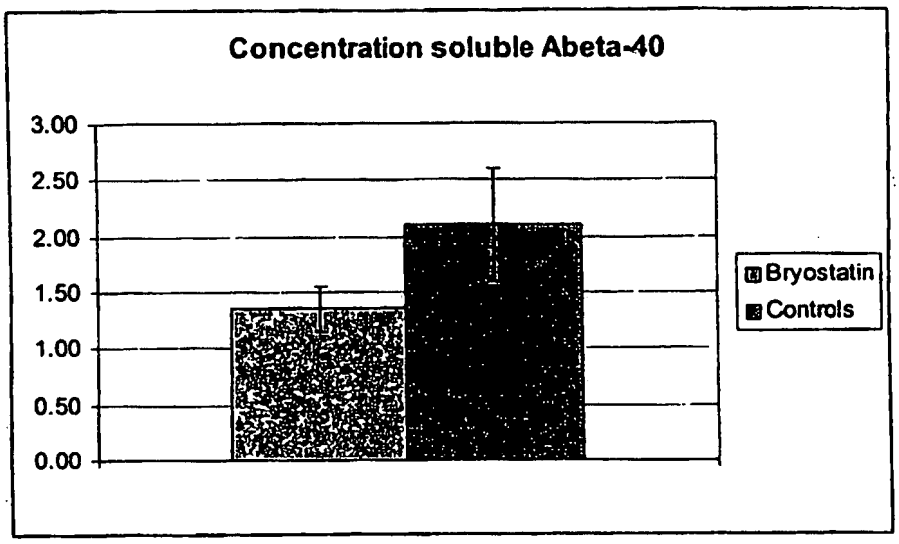

Concentration soluble Abeta-40
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Bryostatin
Controls

FIG. 12

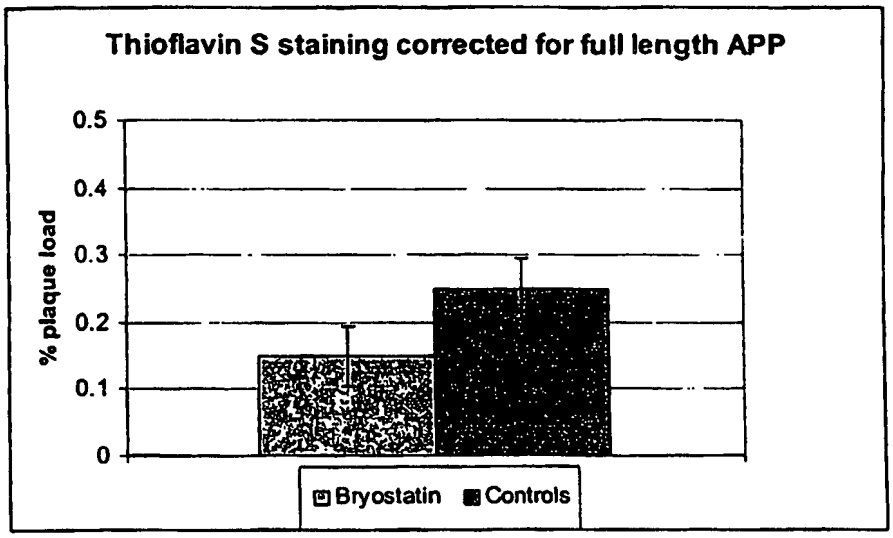

Thioflavin S staining corrected for full length APP
% plaque load
0.5
0.4
0.3
0.2
0.1
0
Bryostatin
Controls

FIG. 14

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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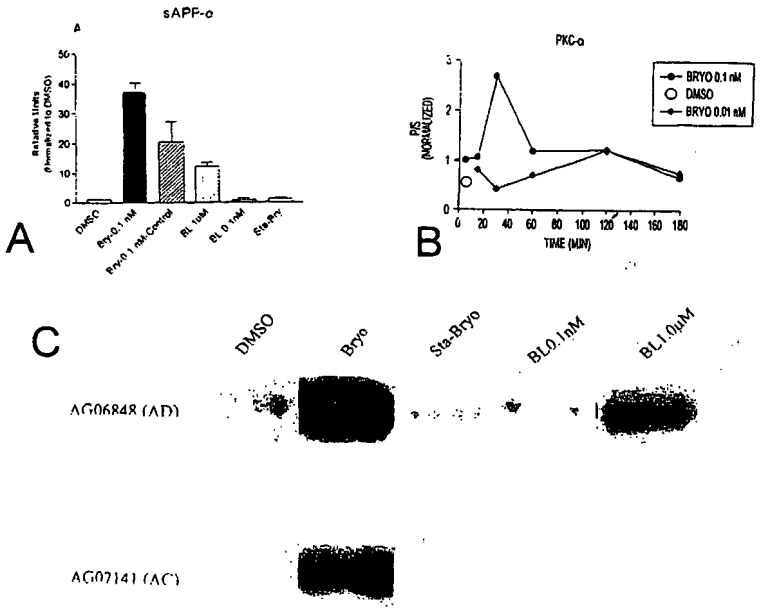
— *with international search report*

[Continued on next page]

(54) Title: PKC ACTIVATION AS A MEANS FOR ENHANCING sAPPα SECRETION AND IMPROVING COGNITION USING BRYOSTATIN TYPE COMPOUNDS



(57) Abstract: The present invention relates to compositions and methods to modulate α-secretase and/or to improve cognitive ability. The invention further relates the improved/enhanced cognitive ability in diseased individuals, particularly Alzheimer's Disease patients, and treatment thereof through increased sAPP production. Macrocyclic lactones (i.e. bryostatin class and neristatin class) are compounds preferred for use with the present composition. The present invention also provides methods for increasing the generation of non-amyloidogenic soluble APP comprising the activation of protein kinase C (PKC) by administering an effective amount of PKC activator(s).

WO 2004/004641 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/20820

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/35,31/335
US CL : 514/449-453

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/449-453

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP 2001240581 A (CHOI et al) 04 SEPTEMBER 2001 (04.09.2001) the entire document, particularly, the abstract, and the claims.	1-36
Y	JP 06279311 A (TAMURA et al) 04 OCTOBER 1994 (04.10.1994) the entire document, particularly, the abstract, and the claims.	1-36
Y	ZHANG et al 'Preclinical pharmacology of the natural product anticancer agent bryostatin 1, an activator of protein kinase C," Cancer Research. 15 February 1996, vol. 56, pages	1-36
Y	IBARRETA et al. 'Benzolactam (BL) enhances sAPP secretion in fibroblasts and in PC12 cells,' Ageing, April 1999, vol 10, no. 5, pages 1035-1040	1-36
Y	US 6,407,058B1 (STADDON et al) 18 JUNE 2002 (18.06.2002), the entire document, particularly, the claims.	1-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search
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